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14. ABSTRACT

The overall goals of the VISION project are to identify new neuroprotective agents in mouse models of injury to the visual axis and propose therapeutic candidates for preserving vision in warfighters who have sustained ocular injuries. In our first year of this project, we have recruited and hired knowledgeable and dedicated scientists to establish the infrastructure necessary for the proposed studies. During this first year, we established: (1) animal model and histology core laboratories, (2) a sophisticated data management system, (3) all 3 models of injury to the visual axis, (4) techniques for the quantitative assessment of injury-induced damage to visual axis tissues, and (5) in vitro assessment of 4 novel neuroprotective agents. We are now ready to test these neuroprotective agents in our models and will identify additional neuroprotective pathways in Year 2 of this project.

15. SUBJECT TERMS
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INTRODUCTION:

This is the first annual report for the VISION (Vision Integrating Strategies in Ophthalmology and Neurochemistry) project at UNTHSC. We currently have 6 PIs, 6 postdoctoral fellows, 5 graduate students, and 3 research technicians actively involved in this very ambitious project. In combat situations, traumatic eye injuries are frequent, leading to irreversible damage to the visual axis. The overall goal of the VISION project is to discover neuroprotective strategies in three separate mouse models of injury to the visual axis, in order to identify potential candidates for the treatment of combat eye injuries and preserve vision in our injured warfighters. We have established three different mouse models of ocular injury with different injury-initiating mechanisms (i.e. optic nerve crush, retinal ischemia/reperfusion, and chronic ocular hypertension). We have developed techniques to quantify damage to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus) that are damaged in these three models. We are testing neuroprotective agents and strategies, including neuroprotective estrogens, sigma-1 agonists, Brn3b, and inhibitors of protein stress to determine their efficacy in protecting the retina, optic nerve and superior colliculus from the damage induced by each of the 3 models. In addition, we are also evaluating time dependent, injury-induced changes in gene expression in the effected tissues to identify the major pathogenic pathways involved in order to develop new therapeutic approaches for neuroprotection and neuroregeneration. In the following report, we highlight the considerable progress made in this first year.

BODY:

Personnel: We have recruited and newly hired 10 scientists and 5 graduate students to support this very ambitious project.

Dr. Marina Gorbatyuk was recruited as a new Asst. Professor and has become the 6th PI for this project. Dr. Gorbatyuk has a proven record on using viral vector gene therapy to functionally and structurally rescue photoreceptor cells in rodent models of retinal degeneration. She brings expertise in gene therapy and in cellular stress due to protein misfolding. The unfolded protein stress response plays important roles in neurodegeneration pathogenesis, and we have preliminary data suggesting that this also may occur in our models of ocular injury. We also will use viral gene delivery to retinal ganglion cells to validate newly discovered pathogenic pathways.

We have recruited and hired 6 postdoctoral fellows to support this project. Three of the postdocs (Zhang Zhang, PhD; Byung-Jin Kim, PhD; Yang Liu, PhD) are responsible for establishing the 3 experimental mouse models (see progress below) and quantitatively assessing damage to the retina, optic nerve, and superior colliculus in the brain. Everett Nixon, PhD is working with PI James Simpkins, PhD to screen and characterize neuroprotective estrogens in order to select the best candidates for in vivo testing. Dorota Stankowska, PhD is working with PI Raghu Krishnamoorthy, PhD to clone Brn3b into expression vectors and prepare AAV viruses to test the neuroprotective and neuroregenerative capablilies of Brn3b in our 3 animal models. Xiao Qin Wang, PhD will be working with PI Marina Gorbatyuk to evaluate the neuroprotective roles of viral vectors targeting the protein stress pathway.

We hired 3 research technicians to help with the animal models and with histological assessment of damage to the retina, optic nerve, and superior colliculus. Sandra Neubauer is a trained histology technician who has set up and runs our histology core facility. Holly Tebow and Terri Beckwith are help run all 3 animal models as well as assessment of damage to the retina, optic nerve, and superior colliculus.

We have 5 graduate (PhD) students working on the VISION project. Wanda Medina is working with Dr. Clark to study ocular and brain injury associated extracellular matrix remodeling (fibrosis and glial scar formation. Tasneem Putliwala is working with Dr. Clark to harvest RNA from the retina, optic nerve head, optic nerve, and superior colliculus at different time points in all 3 mouse models and will perform an extensive genomics study in order to identify pathogenic pathways and new potential therapeutic targets. She will collaborate closely with our U. Iowa bioinformatics colleagues for rigorous pathway analysis. Sean Silverman is working with Dr. Clark to evaluate the potential pathogenic role of the complement pathway in damage to the retina, optic nerve, and superior colliculus in all 3 mouse models. Nitasha Phatak is working with Dr. Krishnamoorthy to study the role of Brn3b in optic nerve regeneration in all 3 mouse models. Brett Mueller is a PhD, medical student working with Dr. Yorio to determine the neuroprotective effect of sigma-1 receptor agonists.

Establishing CORE Laboratories to Support Overall Project:

Animal Models Core: We now have 3 postdocs, 2 research technicians, and a graduate student involved in setting up and running the 3 models of ocular and brain injury. In April, we visited the laboratory of Dr. Rob Nickells at U. Wisconsin to learn the techniques for: optic nerve crush in mice, Nissl staining of retinal flat mounts, and quantitative analysis of cells in the retinal ganglion cells layer. We now have perfected and are routinely running these techniques (see results below). In April, we also visited the previous laboratory of Dr. Abe Clark at Alcon Research, Ltd. to learn the techniques of non-invasive IOP measurement in conscious mice and intravitreal injection of viral expression vectors,

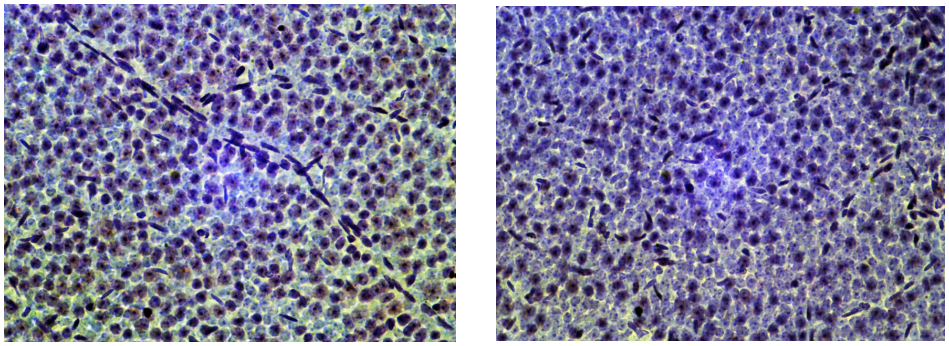
which are essential for the mouse model of chronic ocular hypertension. We have successfully employed these techniques to routinely run our model of chronic ocular hypertension (see results in the following section). We have run preliminary studies on the retinal ischemia/reperfusion mouse model and determined that 60 minutes of ischemia (via intracameral cannulation of the anterior segment at 120 mmHg) followed by reperfusion caused retinal damage assessed by changes in retinal thickness. We purchased two new machines to evaluate retinal structure and function in live mice. UNTHSC provided funds ($140,000) to purchase a spectral domain optical coherence tomography (SD-OCT) instrument, which provides close to histological resolution of the layers of the retina in mouse eyes. We used VISION funds to purchase an electroretinogram (ERG) device that measures the functional response (generation of electrical currents) to light stimulation of the retina. Once validated by correlating OCT and ERG measurements with our histological assessments of damage, we should be able to longitudinally follow retinal damage in all three of our mouse models.

Histology Core: We hired Sandra Neubauer, a trained histology technician to set up and run our Core histology lab. This laboratory supports all histological assessments of damage to the retina, optic nerve, and superior colliculus for all three of our mouse models of ocular/brain injury. New equipment was purchased to support tissue processing, paraffin embedding, sectioning, staining of sections, and photomicroscopy. In addition, we recently received a new cryostat that was donated to our histology laboratory by Alcon Research, Ltd. We will upgrade this new cryostat with 2nd year equipment funds.

Data Management System: We are collaborating with the bioinformatics group at the University of Iowa, headed by Dr. Terry Braun, to develop a data management system to support the VISION project. Our VISION project will involve the use of more than a thousand mice and the generation of hundreds of thousands of histology images as well as statistical and genomics data. The Iowa group purchased and set up 8 data management system workstations, for the laboratory of each PI as well as for the animal model and histology core laboratories. We evaluated and tested the Jackson Laboratory mouse colony management system (JAX CMS), which has been loaded on each workstation and will be used to order and follow each mouse and each mouse sample being used in the VISION project. The JAX CMS will also be used to plan, organize, and communicate all aspects of the animal models as well as the assessment of damage and neuroprotective efficacy. Image J software has been loaded on each workstation to support image analyses and quantification of tissue damage. All images and data generated will be specifically annotated and archived on a central server with multiple Terabytes of storage capacity. This central sever is frequently backed up so that we will not loose any of the valuable data generated from this project. All images and data will be readily searchable by members of the VISION team via a clear set of annotations.

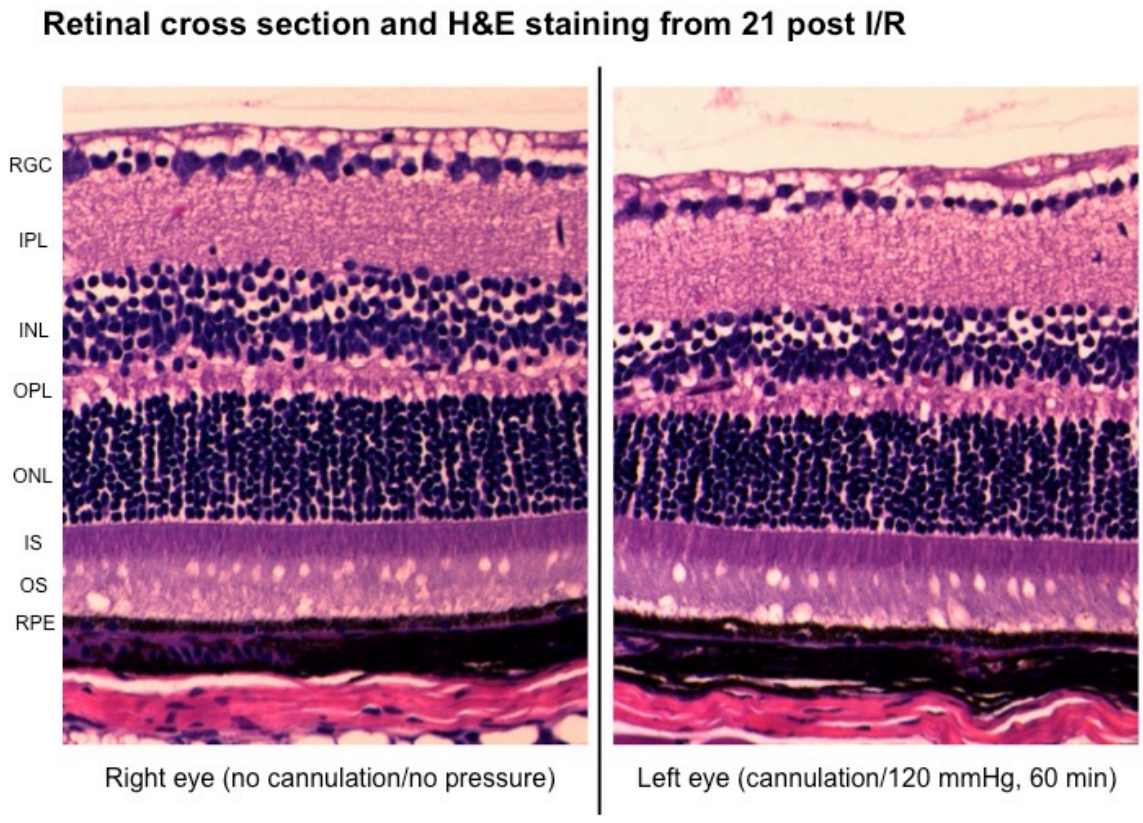
Assessment of Damage: We have developed quantitative techniques to visualize and quantify damage to the retina, optic nerve, and superior colliculus for each mouse used in our three models of ocular/brain injury.

Counts of Cells in Retinal Flat Mount: There will be progressive loss of retinal ganglion cells (RGCs) in each of the 3 models of ocular/brain injury. At each time point of the studies, eyes are enucleated from humanely sacrificed mice, gently fixed, and the retinas are carefully removed and “flat mounted” RGC layer up on microscope slides. The retinas are stained with Nissl stain, and the cells in the RGC layer are counted in four sections from each quadrant of the retina. There are approximately equal numbers of RGCs and displaced amacrine cells in this retinal cell layer, so total loss of RGCs would equate to a 50% decrease in cells in this cell layer. An example of Nissl stained retinas is shown below.



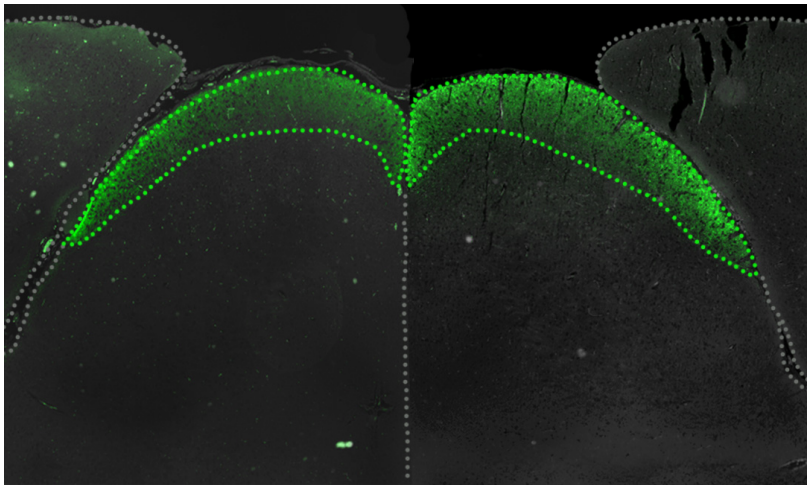
Nissl stained retinal flat mounts showing cell nuclei in the retinal ganglion cell layer. **Left Panel**: Control retina. **Right Panel**: Retina 28 days after optic nerve crush injury. There is a significant loss of RGCs in the ONC retina compared to the control retina. RGCs make up approximately 50% of the cells in the retinal ganglion cell layer, with displaced amacrine cells as the remaining cells that are not affected by ONC injury.

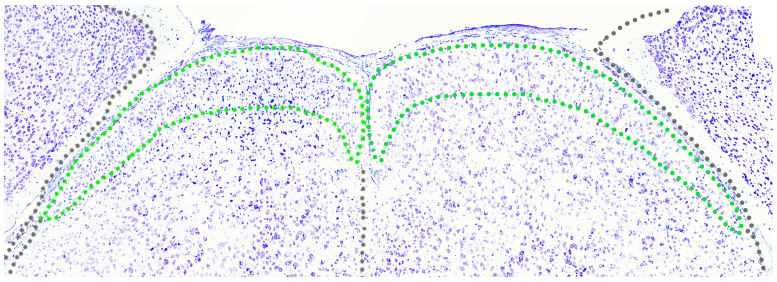
Retina cross-sections: Ischemia reperfusion (I/R) injury to the retina will not only damage RGCs, but also damage cells in other retinal cell layers. To quantify retinal damage in this model, we will measure changes in the overall retinal thickness as well as changes in the thickness of the various retinal cell layers. We also will be able to count the number of cells in the RGC layer to provide an additional quantitative assessment of damage in this model. Below is an example of retinal cross section images showing the various retinal cell layers 21 days after retinal ischemia/reperfusion injury (Left panel = control eye; Right panel = I/R eye).



Coronal Sections of the Superior Colliculus (SC): Approximately 90% of the RGC axons target the cortical neurons in the superior colliculus in the brain. In order to identify the region targeted, we

injected a construct of Alexa conjugated cholera toxin B subunit (CTB) into the vitreous of one eye, and 24-48 hours later sacrificed the mouse to examine Alexa staining in the SC. The CTB tracer is taken up by RGCs and anterograde transported through the optic nerve to the axon termini in the contralateral SC. We performed this injection in left and right eyes to shown the pattern of labeling in both hemispheres. The labeled regions were outlined across all 20 SC sections and a template was prepared to superimpose on Nissl stained specimens. This template is used to count neurons in the targeted areas to assess injury-induced damage to the SC.

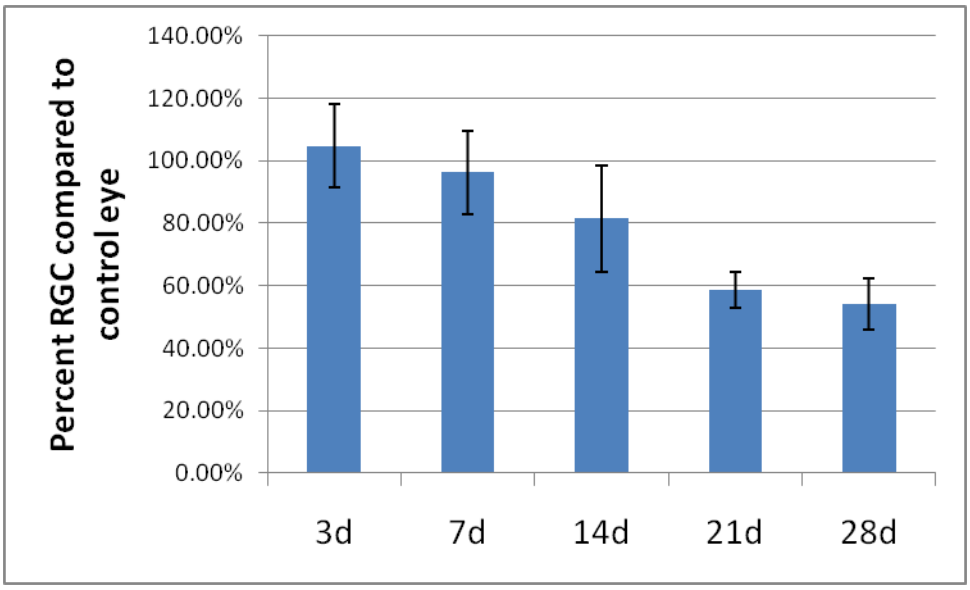




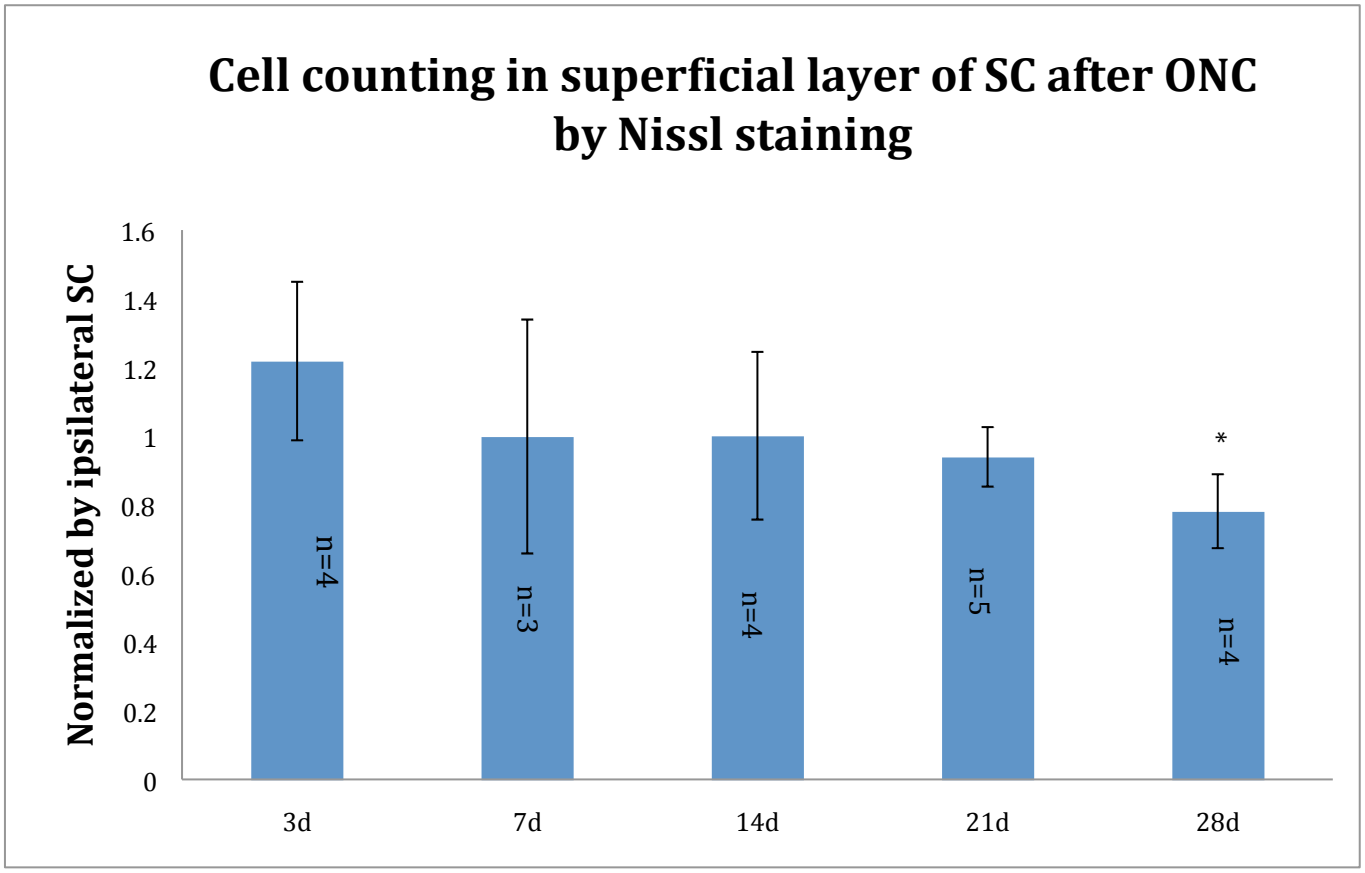
Coronal sections through the superior culliculus (SC). ***Above image:*** Alexa labeled cholera toxin B (CTB) was injected into the vitreous of one eye and 48 hours later, anterograde transport from the eye to RGC axon terminals in the brain was identified in SC sections. Labeled regions were outlined to generate templates to use in Nissl stained SC sections. The image on the left is from a CTB injection into the right eye. The image on the right is from a CTB injection into the left eye. ***Below Image:*** Nissl stained SC coronal section. CTB templates were used to identify regions of SC that is innervated by RGC axons. Neuronal cells are counted within this boundary to assess damage to this visual center of the brain.

Progress in Models of Ocular/Brain Injury: We have established 3 different mouse models of injury to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus): optic nerve crush, choonic ocular hypertension, and retinal ischemia/reperfusion.

Optic nerve crush (ONC): We have run a time course to quantify damage to RGCs and the superior colliculus after unilateral ONC. There is progressive and statistically significant loss of cells in the RGC layer, assessed by Nissl stained retinal flat mounts (see below). The 50% loss that occurs at 28 days post-ONC would equate to an almost total loss of RGC because half the cells in this layer are displaced amacrine cells.

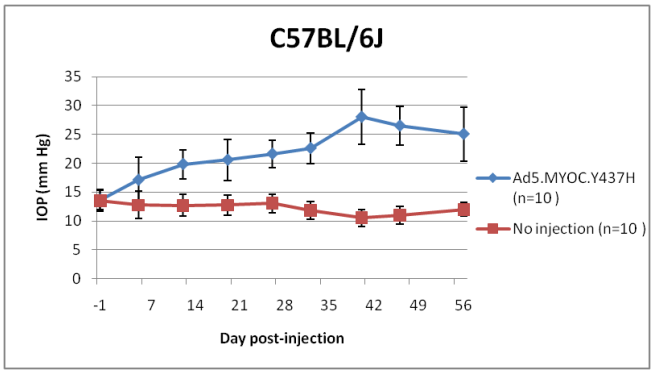


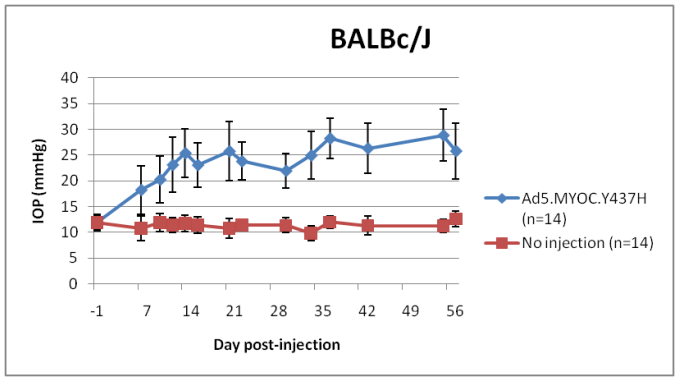
In addition to loss of cells in the RGC layer, we also observed a statistically significant loss of neurons in the region of the SC innervated by RGC axons at 28 days post-ONC (see below).

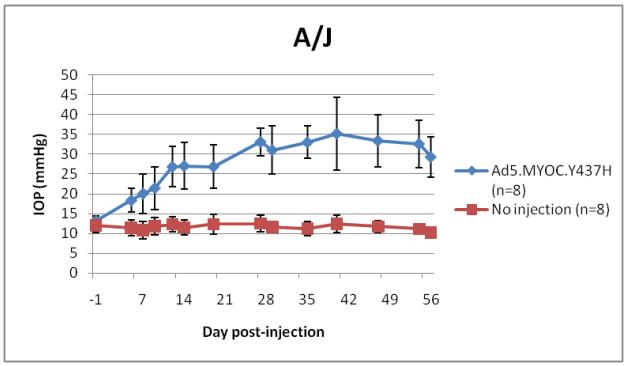


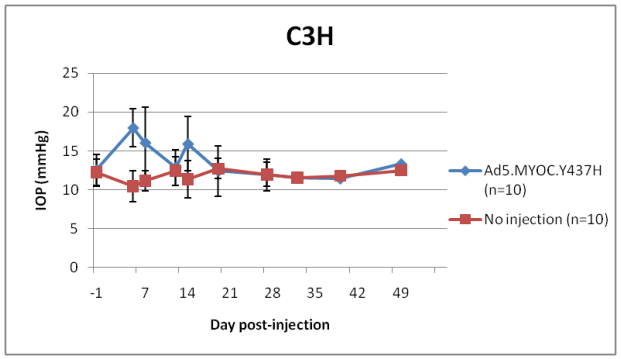
We have harvested retinal, optic nerve head, optic nerve, and SC tissues at various time points for RNA extraction and gene expression profiling in order to identify and characterize pathogenic pathways. We are in the process of extracting RNA fro these samples, which will be sent to the DNA Core facility at U. Iowa for analysis of gene expression using Affymetrix Mouse GENE arrays. We have preliminary gene expression data implicating activation of the unfolded protein response and protein stress in this model.

Chronic ocular hypertension: We performed a small strain survey to identify which mouse strain was more susceptible to elevated intraocular pressure (IOP) induced optic nerve damage. Mice were injected unilaterally with an adenovirus expression vector encoding a glaucomatous mutation in the Myocilin gene (Ad5.MYOC.Y437H), and IOPs were measured non-invasively measured in conscious mice from days -1 to 56 days post-injection. This vector transduces the anterior segment of mouse eyes and over-expresses mutant myocilin. IOPs were significantly elevated in 3 of the 4 mouse strains evaluated (C56BL/6J, BALBc/J, and A/J strains (see below).

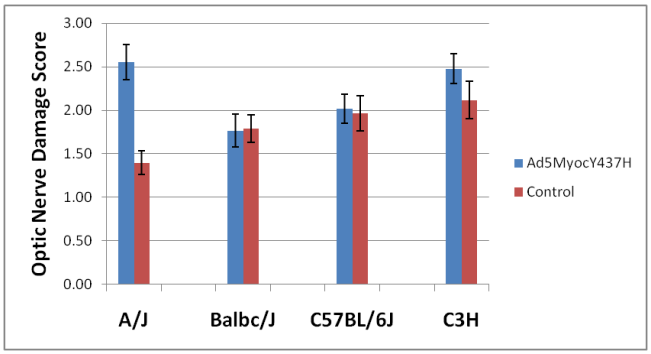


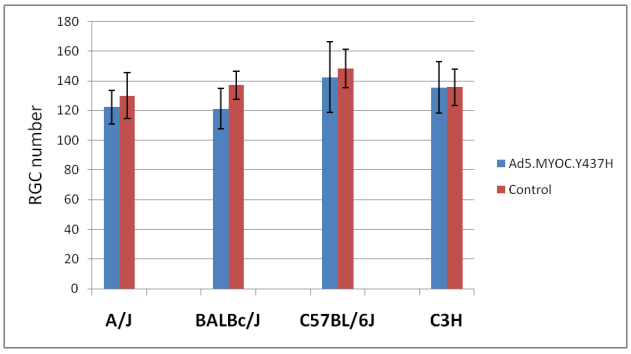




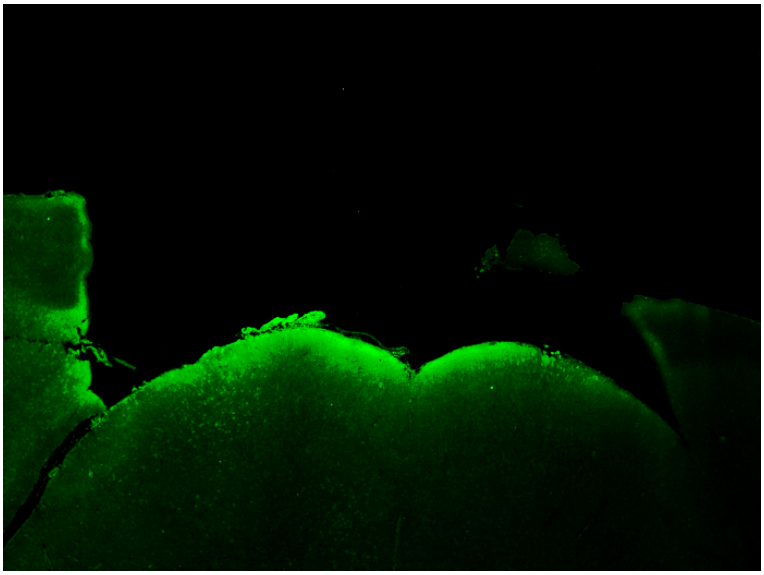


However, only the A/J strain developed significant optic nerve damage at the 8 week time point evaluated (see below). Optic nerve damage was assessed by PPD staining of optic nerve cross-sections and clinically graded with an optic nerve damage scores ranging from 1 (no damage) to 5 (> 90% axons damaged or lost). The optic nerves of hypertensive A/J had an average damage score of 2.5, while the damage score in the contralateral optic nerve from the normal eye was 1.4. Although there was significant damage to the optic nerve in A/J mice, there did not appear to be any loss of RGCs. This finding supports the concept that the initial pressure induced damage occurs in optic nerve axons, with subsequent loss of RGCs. We are in the process of performing a detailed time course study, evaluating damage from 0-24 weeks.



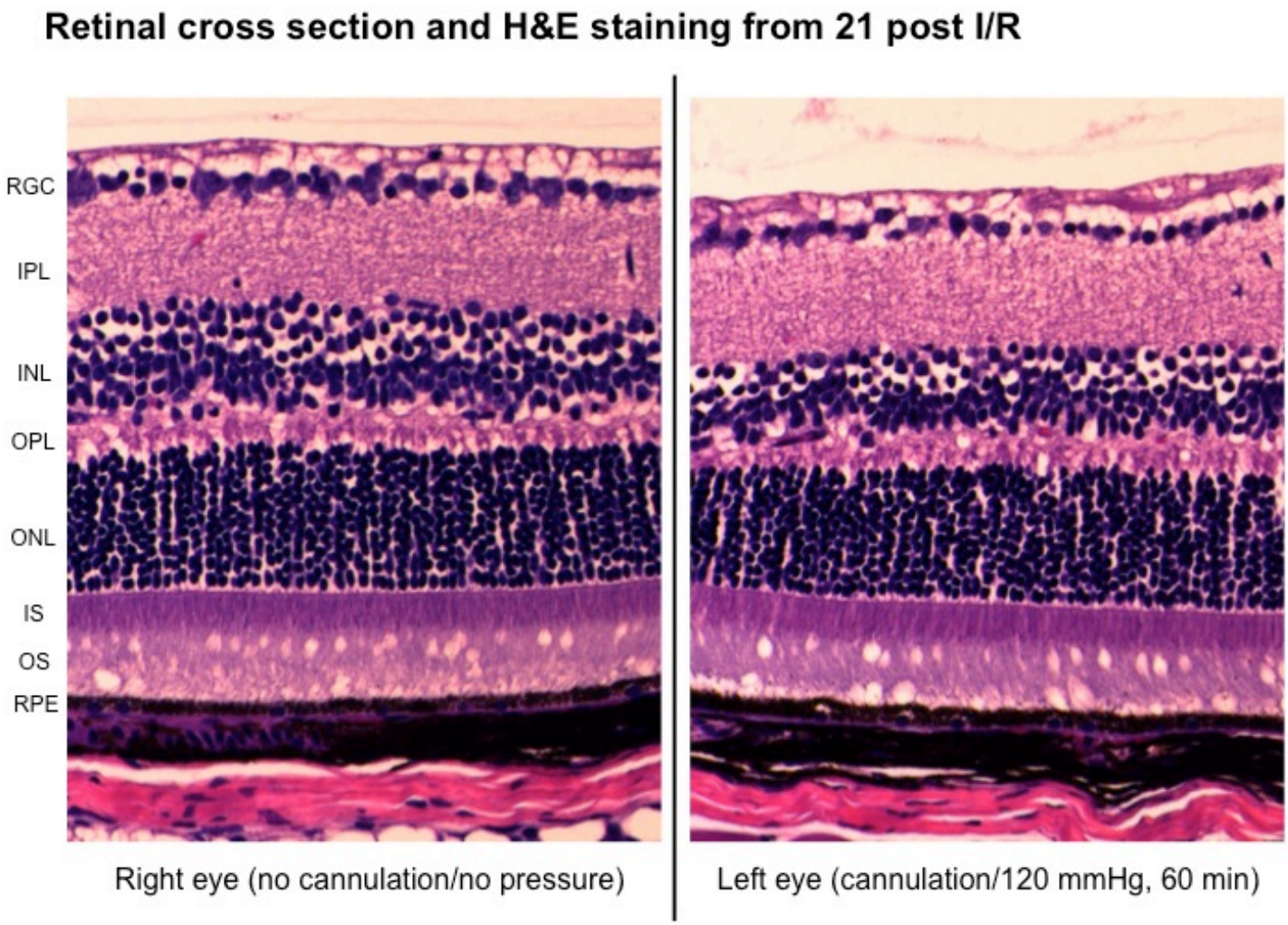


Complement activation has been implicated in early damage and loss of RGCs during developmental pruning as well as in chronic pressure-induced neuropathy. We evaluated the potential activation of complement component C1q in the superior colliculus (SC) of mice 2 weeks after generating ocular hypertension. There appears to be more C1q immunostaining in the contralateral SC (the right eye was hypertensive) after only two weeks of elevated IOP (see below).



Retinal ischemia/reperfusion: We have set up the model of retinal ischemia/reperfusion injury in mice by carefully cannulating the anterior segment of one eye per mouse and raising the fluid filled reservoir to 120 mmHg (above systolic blood pressure). This blocked blood flow to the retina, which could clearly be seen using an ophthalmoscope. After 45 or 60 minutes of ischemia, the needle was removed from the eye and blood flow to the retina restored (i.e. reperfusion). Controls consisted of the contralateral unmanipulated eye and a second group of animals that had one eye cannulated for the same duration, but pressure was not raised (and therefore blood flow not blocked). The animals are sacrificed at 7, 14, 21, and 28 days to assess damage to the retina, optic nerve and superior colliculus. Pilot studies showed that 45 minutes ischemia did not appear to damage the retina, assessed by histological examination of retinal cross-sections, over this time course (data not shown). However, exposure to 60 minutes of ischemia appeared to damage the retina as seen by a decrease in retinal thickness at 21 days (see below). We are in the process of repeating this study with additional mice and performing a quantitative assessment of damage to the retina (thickness of retinal layers and cell counts in the RGC layer), optic nerve (see above) and superior colliculus (see above).

Experimental set-up for the mouse ischemia/reperfusion model of ocular and brain injury. Anesthetized mice are placed on a heating pad to maintain body temperature throughout the procedure. A single eye from each mouse is carefully cannulated with a needle connected to a raised fluid reservoir equivalent to 120 mmHg. The elevated IOP is maintained for 60 minutes, and an ophthalmoscope is used to confirm retinal ischemia. After 60 minutes, the needles are removed from the eyes and blood flow is restored (i.e. reperfusion).



H&E stained retinal cross sections from an experimental mouse 21 days after exposure to retinal ischemia/reperfusion injury (pilot study). The left eye was exposed to ischemic injury (120 mmHg for 60 minutes) while the right eye was unmanipulated and served as a control. There appears to be a decrease in inner retinal thickness in the left eye. We are in the process of repeating this study to perform quantitative assessments of damage over time.

Neuroprotection strategies:

Neuroprotective estrogens (Simpkins):

This year, we have focused on optimizing compounds and assessing them in relevant retinal cell lines. To date, we have completed a series of studies showing that several of our non-feminizing estrogens are protective in the RCG-5 retinal cell line. These studies show that 17 β-estradiol (E2) as well as ZYC-3 (2-adamantyl-estradiol) and ZYC-26 (2-adamantyl-4-methl estrone) are potent neuroprotectants against glutamate toxicity.

We then assessed ZYC-3 in a 661W retinal cell line. We observed that glutamate caused a dose-dependent cell death. We observed the non-feminizing estrogen, ZYC-3, was potently neuroprotective against glutamate (at its LD50) induced-cell death. To determine if the non-feminizing estrogens exert

their neuroprotective effects through a membrane estrogen receptor, we attempted to protect cell using the membrane estrogen receptor agonist, G1, and demonstrated no protection against glutamate toxicity. We are now assessing the signaling pathways that ZYC-3 employs to provide protection in 661W cells, including protecting against excessive calcium influx, reactive oxygen species production, and mitochondrial destabilization.

Currently, we are optimizing the assay conditions for a determination of the dose-dependence of the effects of ZYC-3, ZYC-26 and E2 in 661W cells using an LDH assay and a calcien AM assay. Following the establishment of these assays in this cell line, we will apply the same procedures to a primary retinal ganglia cell line, on which we are working to set up in the laboratory.

Targeting the protein stress response (Gorbatyuk):

To develop new neuroprotective and regenerative strategies for treating ocular injuries and degenerative ocular diseases, we have designed adeno-associated viral vectors containing expression cassettes for therapeutic genes. Search for therapeutic agents in a Pubmed have indicated that ER resident chaperone BiP/GRP78, Heat Shock Factor 1 (HSF1) could be potential candidates.

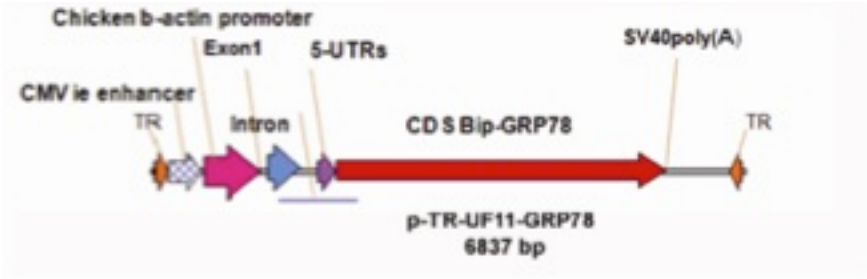


Fig. ? Design of AAV vector expressing the human BiP/GRP78. The expression of BiP is driven by the chicken β-actin promoter. The processing of the BiP mRNA is facilitated by the insertion of the chicken β-actin exon 1 and intron. The SV40 poly A is present for stabilization, maturation and for further BiP export from the nucleus.

Justification of Choice for Therapeutic Genes. BiP as therapeutic molecular chaperone with a cytoprotective role. The main function of BiP is to facilitate correct protein folding in the ER. BiP is found to: (1) play an essential role in the biosynthesis of proteins; (2) maintain the permeability barrier of the ER during early stages of protein translocation; (3) target misfolded proteins for proteosomal degradation (via ERAD) and (4) serve as a sensor for ER stress.

Over-expression of BiP protects cells from apoptosis by an association with pro-caspase-7. It has also been demonstrated that over-expression of BiP protein reduces net flux of Ca2+ from ER to mitochondria, increases Ca2+ uptake capacity in isolated mitochondria, reduces free radical production, and preserves respiratory activity and mitochondrial membrane potential after stress protecting astrocytes from ischemic injury (Ouyang et al., 2010).

Therefore, full length cDNA of human BiP/GRP78 (NM_005347) was inserted in an AAV plasmid with serotype 2 terminal repeats. The expression was controlled by the chicken β-actin promoter and CMV immediate early enhancer (the CBA promoter). BiP mRNA was processed from the long transcript by splicing of the intron following the first β-actin exon, which does not code for protein. The maturation and stabilization of BIP mRNAs were supported by the SV40 polyA signal in the construct. The map of AAV vector expressing BiP/GRP78 cDNA is shown in the above figure.

We have already tested this virus in P23H Rhodopsin rat model of retinitis pigmentosa and found that it has a therapeutic impact on degenerating photoreceptors. The vision test assessed by the scotopic ERG has demonstrated 2-fold increases in a- and b-waves amplitudes (Gorbatyuk et al., 2010).

Heat Shock Factor 1. DNA-binding protein specifically binds to heat shock promoter elements (HSE) and activates transcription. HSF1 exists as an inactive monomer in a complex with Hsp40/Hsp70 and Hsp90. Upon stress, such as elevated temperature, HSF1 is released from the chaperone complex and

trimerizes. HSF1 is then transported into the nucleus where it is hyperphosphorylated and binds to DNA containing heat shock elements (NGAAN). HSF1's target genes include major inducible heat shock proteins such as Hsp70

Recently it has been shown that HSF1-activated compound suppress polyglutamine-induced neurodegeneration through Induction of multiple molecular chaperones such as Hsp70, 40 and 90 (Fujikake at al., 2008). Zhang et al., 2001 have demonstrated that over-expression of HSF1 protects against ischemia/reperfusion injury by diminishing oxidative stress via down-regulation of intracellular ROS generation and inhibition of JNK phosphorylation.

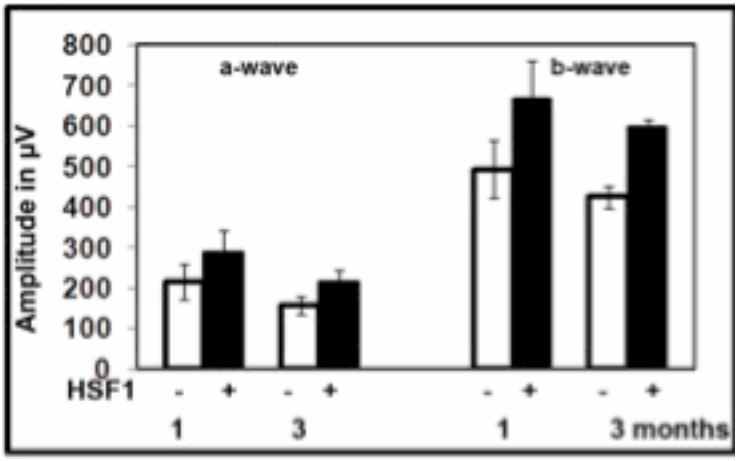


Fig. ? Over-expression of HSF1 in P23H RHO-3 photoreceptors leads to sustained preservation of a- and b-wave amplitudes of the scotopic ERG.

Therefore, the full length of human HSF1 was inserted an AAV plasmid with serotype 2 terminal repeats the way as it was described above.

We have already tested this virus in P23H Rhodopsin rats (Gorbatyuk et al, 2011 in press) and found that over-expression of HSF1 declines retinal denegation (see adjacent figure).

Pro-apoptotic CHOP protein. It was first identified as growth arrest- and DNA damage inducible gene 153 (GADD153), DNA–damage-inducible transcript 3 (DDIT3). It is ubiquitously expressed at very low levels. However, it is robustly expressed by perturbations that induce stress in a wide variety of cells. CHOP is present in the cytosol under non-stressed conditions, and stress leads to induction of CHOP and its accumulation in the nucleus. Induction of the CHOP protein leads to apoptosis. Upon ER stress, the transcriptional induction of CHOP is regulated at least by four cis-acting elements, AARE1, AARE2, ERSE1 and ERSE2. ATF4 binds to AARE1 and AARE2.

In vivo study with CHOP knockout mice and *in vitro* experiments targeting CHOP mRNA by siRNA delivery have demonstrated that reduction of this protein leads to resistance of cells to apoptosis and oxidative stress. Therefore, developing neuroprotective approach for treatment mice models of traumatic eye injury we also want to test siRNAs against this pro-apoptotic gene. Thus, we developed and tested siRNA against CHOP in cultured cells (see above figure).

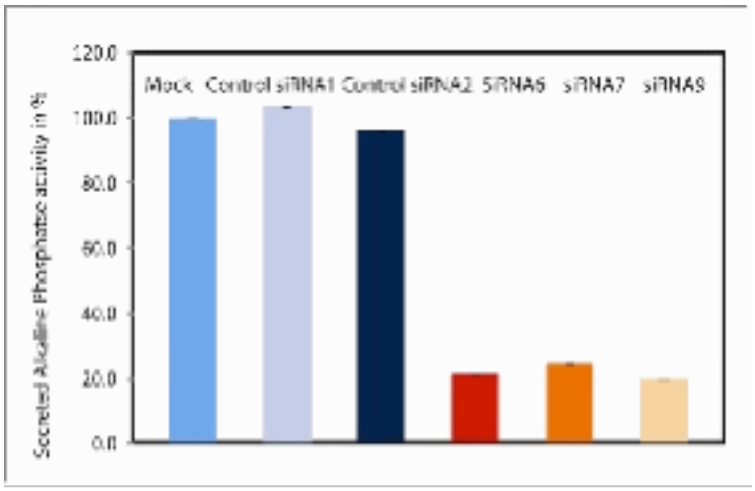


Fig.?. siRNAs targeting mice/rat CHOP mRNA were tested in tissue culture by secreted alkaline phosphatase (SEAP) assay. Mouse CHOP mRNA was inserted in plasmid expressing SEAP. HeLa cells were co-transfected with plasmids expressing SEAP/CHOP mRNAs and control and experimental siRNAs. At 48 hours after transfection cells were harvested to perform SEAP assay by using the QUANTI-Blue™ substrate.

Test siRNA in tissue culture. To test designed siRNAs *in vitro*, HEK 293 cells were co-transfected with plasmids expressing mouse CHOP protein driven by the CMV promoter and synthetic siRNAs. After 48 hours, total cells were analyzed by using SEAP (secreted alkaline phosphatase) assay. An irrelative siRNA was used as a control. Results of experiment are shown above.

For delivery and prolonged expression in animal tissues, the siRNA9 and control siRNA genes were inserted into AAV under control of the human H1 RNA polymerase III promoter and an oligoT terminator sequence. These plasmids also containing the GFP gene driven by the CMV enhancer/chicken b actin (CBA) meric promoter were packaged in AAV5 capsids. Thus, infected cells will express both the siRNA and the GFP-reporter gene.

Targeting the sigma-1 receptor (Yorio):

The goal of this project is to develop methods to protect retinal ganglion cells from noxious stimuli that could potentially cause neuronal degeneration and cell death. The main molecule that is being investigated in this research proposal is sigma-1 receptor. Sigma-1 receptor protect neurons from pathological insults while also promoting neurite outgrowth. Research has demonstrated that the neuroprotective actions of sigma-1 receptors stem from its ability to maintain homeostatic levels of intracellular calcium while also interacting and enhancing mitochondrial function. The purpose of the current grant period is to develop in vitro models for testing potential new agents to protect neurons from degeneration resulting from acute and chronic insults. We have identified a receptor that may have neuroprotection properties and are first testing these mechanisms in cell culture system using isolated primary retinal ganglion cells.

During this first annual report of this contract the following activities were completed:

1. Isolation of primary retinal ganglion cells using method developed by Ben Barres, M.D., Ph.D. (Chair of Neurobiology department at Stanford University School of Medicine). Our lab has demonstrated a high level of pure cultures of retinal ganglion cells (Figure 1A), while also demonstrating robust expression of sigma-1 receptors in those cells (Figure 1B).

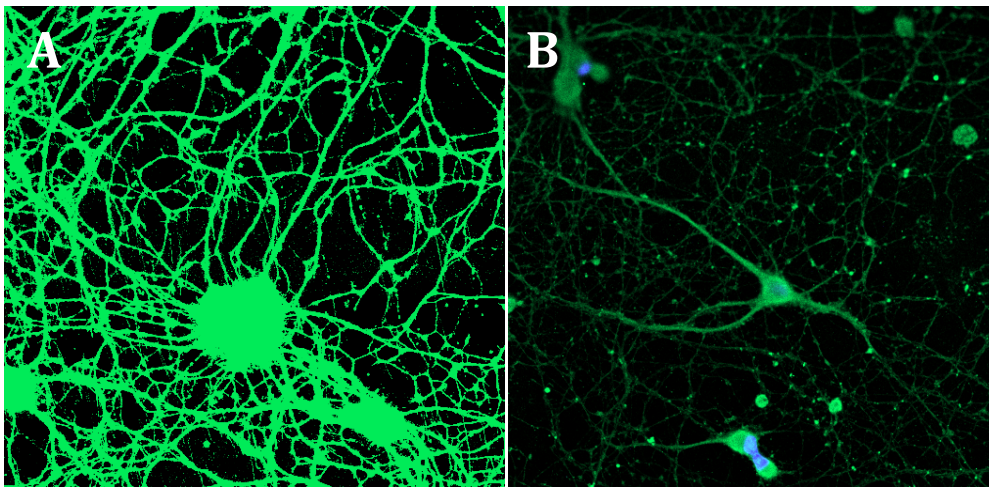


Fig. 1. Purified retinal ganglion cells expressing sigma-1 receptors. (A) Transduced retinal ganglion cells expressing green fluorescent protein demonstrating the robust dendritic outgrowth that is commonly seen in retinal ganglion cells. (B) Immunocytochemistry affirms the presence of sigma-1 receptors in these primary cultures. Sigma – green, DAPI – blue.

2. Demonstrating that an overnight treatment of a sigma-1 receptor agonist on retinal ganglion cells promotes an up-regulation of GluR1, a subunit of AMPA receptor, in the plasma membrane (Figure 2). GluR1 up-regulation in the plasma membrane is commonly associated with dendritic spine out-growth and synaptogenesis.

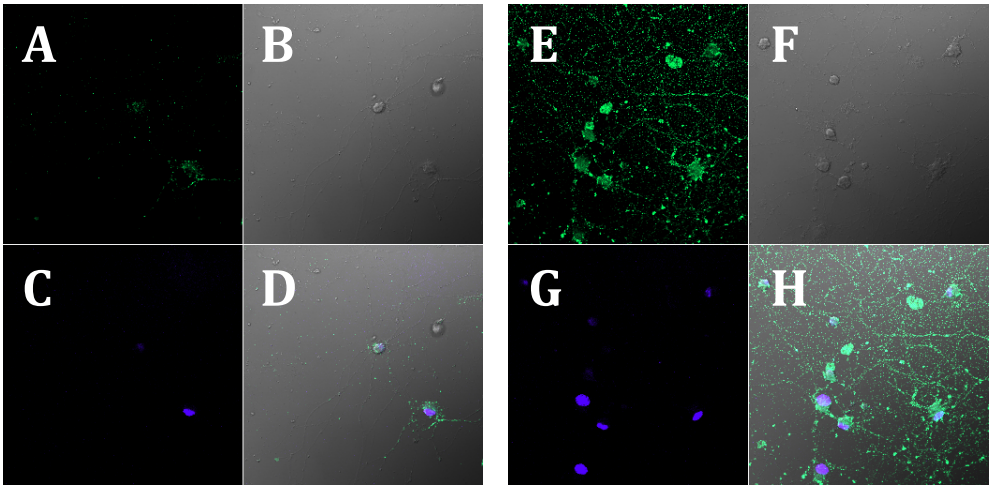
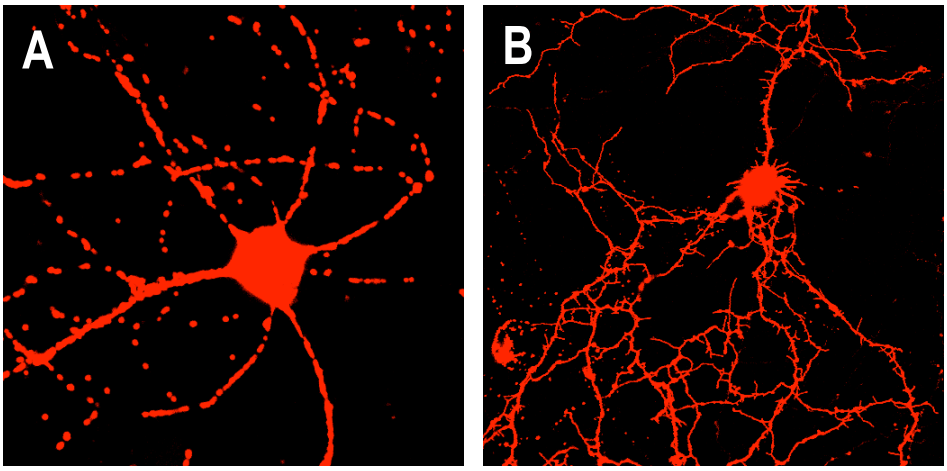


Fig. 2. Immunocytochemistiry on purified retinal ganglion cells expressing GluR1 on the plasma membrane after an overnight treatment with a sigma-1 receptor agonist. (A) GluR1 expression on the plasma membrane after no treatment with a sigma-1 receptor agonist. (B) DIC, (C) DAPI, and (D) Merge of A-C. (E) GluR1 expression on the plasma membrane after an overnight treatment with a sigma-1 receptor agonist. (F) DIC, (G) DAPI, and (H) Merge of E-G.

3. Transfected primary retinal ganglion cells with mitochrondria labeled with "MitoDsRed" (DsRed2 fused to a mitochondrial targeting sequence of cytochrome c oxidase). This construct can be used to study mitochondrial movement, distribution and organelle size within cells. Mitochondria are thought to reside in subcellular regions that require high amounts of energy. In neurons, they have been shown to accumulate in active growth cones. Therefore it is hypothesized that maintaining mitochondrial structure and function is essential to maintaining the overall viability and functionality of retinal ganglion cells during pathological insults. Protecting mitochondrial viability could help in protecting the dendrites and axons of retinal ganglion cells during noxious stimuli. Figure 3A demonstrates a healthy retinal ganglion cell with long, healthy mitochondria projecting throughout the dendrites. When retinal ganglion cells are subjected to 6, 8, and 12 hours of hypoxia (figure 3B), mitochondrial structure deteriorates and begins to wither away from the dendrites of the retinal ganglion cells. It appears that with sigma-1 receptor agonist treatment that mitochondrial structure and distribution is improved when these cells are subjected to 6, 8, and 12 hours of hypoxia treatment. We also have looked at sigma-1 receptor protection of over stimulation of the voltage gated calcium channel (Fig 5)

Without Sigma Agonist

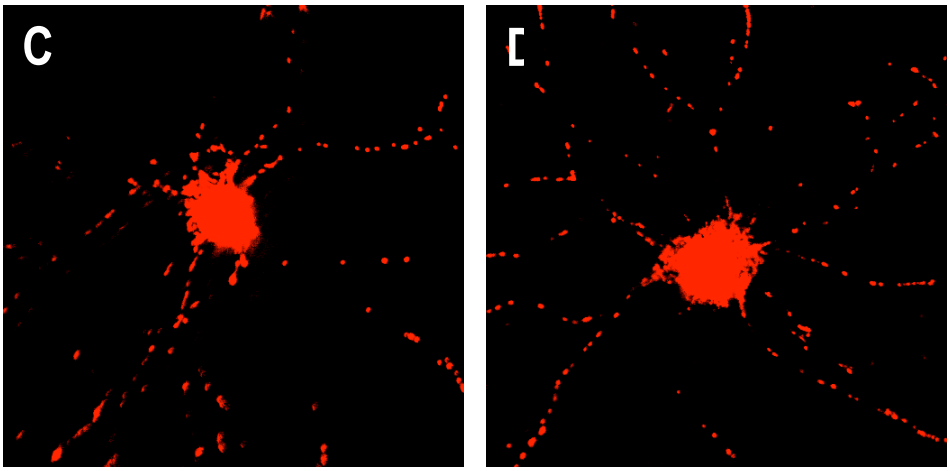
With Sigma Agonist



Control (No Hypoxia)

Without Sigma Agonist

With Sigma Agonist



Hyoxia (6 Hours)

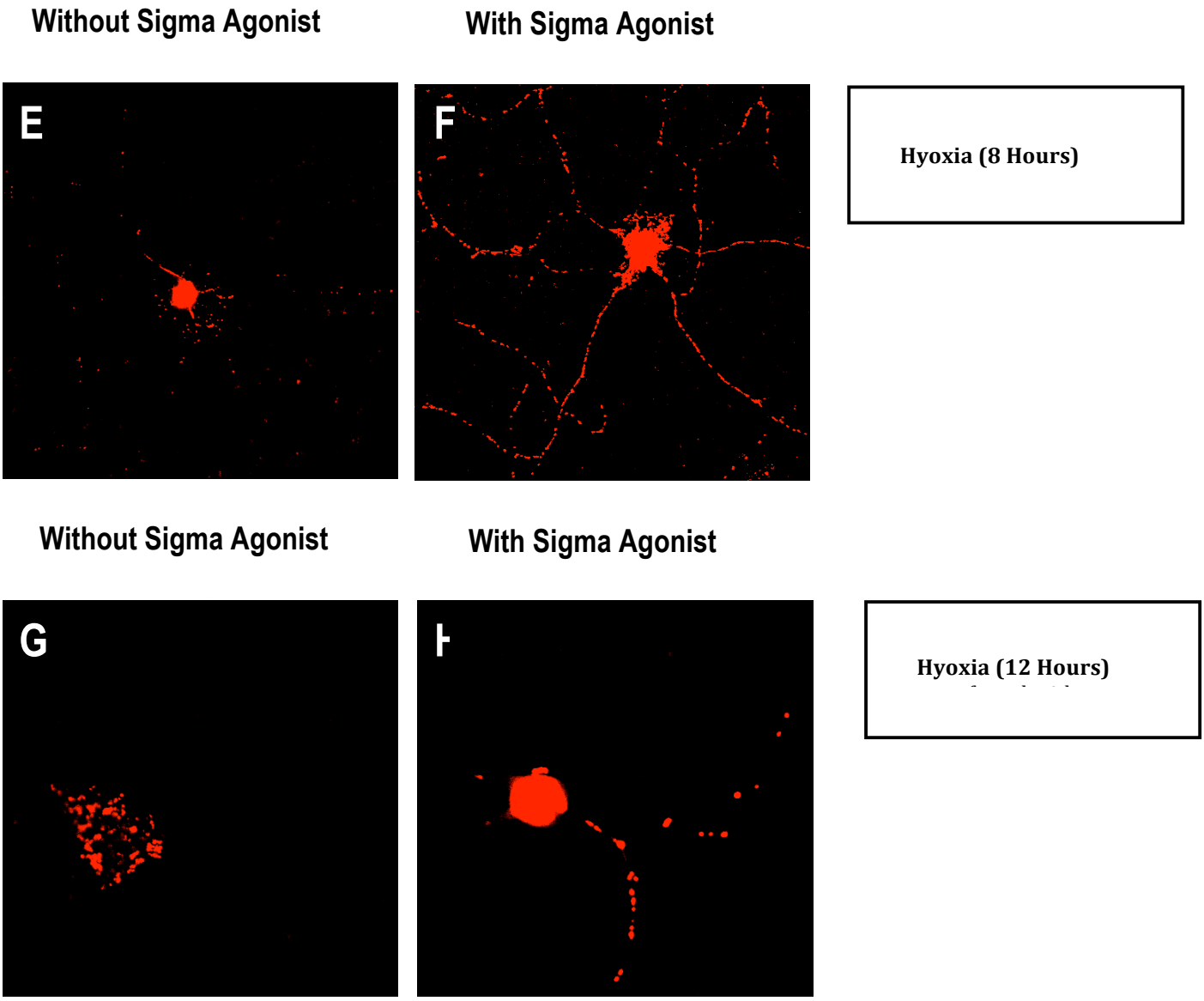
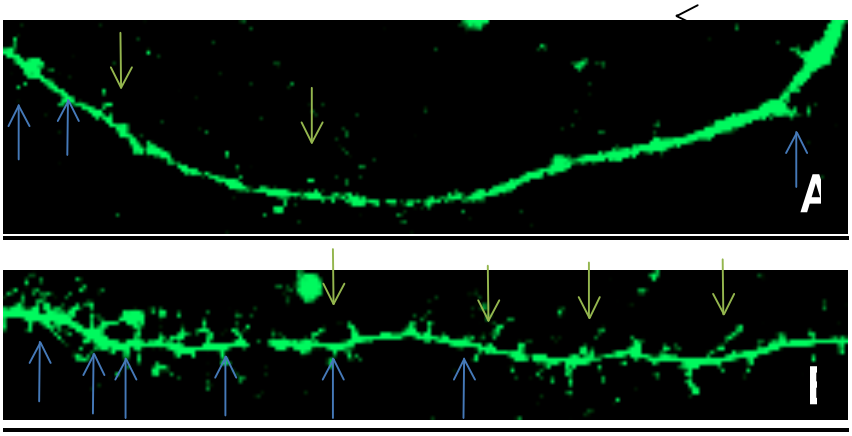


Figure 3. Primary retinal ganglion cells transfected with MitoDsRed were subjected to hypoxia (0.5% oxygen) for 6 , 8 and 12 hours (A-B) Control group that was not subjected to hypoxia which demonstrates fused, large, and healthy mitochondria that are distributed throughout the soma and dendrites. (B) Sigma-1 receptor agonist treated primary RGCs showed a more robust mitochondrial distribution with 12 hours of treatment. (C, E, and G) 6 , 8, and 12 hours of hypoxia treated retinal ganglion cells demonstrated mitochondrial breakdown and fission. (D, F, and H) 6, 8 and 12 hours of hypoxia treated retinal ganglion cells with the administration of a sigma-1 receptor agonist. These cells appear to have improved mitochondria structure and distribution when compared to C, E, and G respectively.

4. Dendritic Spine Analysis:

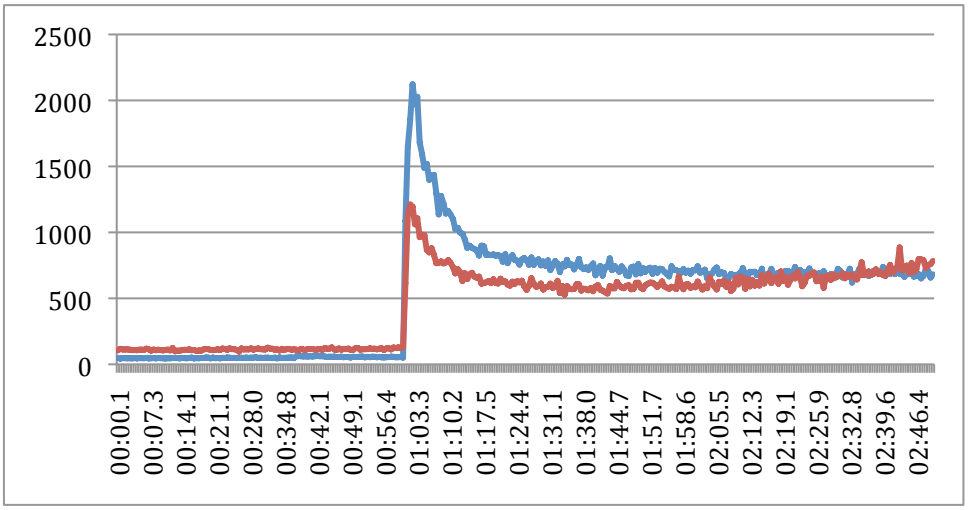


16 Hours Serum Starved w/o Sigma A Sigma Agonist

16 Hours Serum Starved with Sigma Agonist

Figure 4. Close up view of the dendritic structures of primary RGCs. The arrows designate dendritic spine protrusions. All RGCs were transduced with an AAV-GFP construct to enable the viewing of the ultrastructure of the RGCs. RGCs were serum starved with no growth factors for 16 hours in the absence (A) or presence of 1µM of pentazocine (sigma-1 receptor agonist) (B). More spiny projections can be viewed along the dendrites after the administration of a sigma-1 receptor agonist. These dendritic

5. Calcium Influx with Kcl Stimulation in Primary RGCs



--- Potassium Chloride Stimulation without sigma-1 agonist
--- Potassium Chloride Stimulation with sigma-1 agonist

Figure 5. Calcium imaging results have shown that s1r ligand, (+)-N-allylnormetazocine hydrochloride [(+)-SKF10047] in primary RGCs inhibited the influx of calcium through VGCCs by 37% ($p<0.05$). Pretreatment of RGCs with a s1r antagonist, BD10047, potentiated the calcium influx through VGCC by 44% ($p<0.05$).

Targeting Brn3b (Krishnamoorthy):

The overall goal of the project is to determine if a viral vector encoding transcription factor Brn3b is capable of promoting regeneration of damaged axons of the optic nerve. The first step in this project was to set up and optimize protocols for preparing an adeno-associated viral vector encoding the POU domain transcription factor, Brn3b (POU4F2). To achieve this, the following experimental strategies have been successfully carried out in the laboratory:

- A full length cDNA construct encoding the POU domain transcription factor Brn3b was subcloned into the AAV-MCS expression vector (Stratagene), to create the Brn3b expression vector, AAV-Brn3b. The cloning of the Brn3b cDNA was verified by restriction digestion using the appropriate restriction enzymes. Maxipreps of the recombinant plasmids were prepared and purified by centrifugation through CsCl gradients.
- The AAV-Brn3b plasmid was transfected into the 661W neuronal cell line and immunoblot analyses were performed to determine if there was increased expression of transcription factor Brn3b **(Figure 1)**. An increased expression of Brn3b was seen in 661W cells tranfected with AAV-Brn3b, compared to cells transfected with the empty vector (AAV-MCS), suggesting that the expression vector was capable of expressing and upregulating Brn3b expression in the transfected cells.
- Immunocytochemical analyses was performed to determine the effect of overexpression of Brn3b in the 661W cell line. Overexpression of Brn 3b increased neurite outgrowth in 661W cells **(Figure 2)**. GAP43, a neuron-specific cytoplasmic protein which is highly expressed in the growth cones, was upregulated in 661W cells overexpressing Brn3b, compared to cells transfected with the empty vector(AAV-MCS). GAP43 plays an important role during neurite formation and neuroregeneration and immunostaining with GAP43 is often used as an index of neuroregeneration in various *in vivo* experimental models of optic nerve damage.

Further work will be aimed at packaging the recombinant virus encoding Brn3b by cotransfecting the AAV293 cell line with AAV-Brn3b and other components needed for the virus production including pHelper and pAAV-RC (encoding the replication and capsid proteins). The viral particles will be further purified by centrifugation through iodixanol gradients and the viral titer determined using the method described by the manufacturer of the AAV-Helper-Free system (Stratagene).

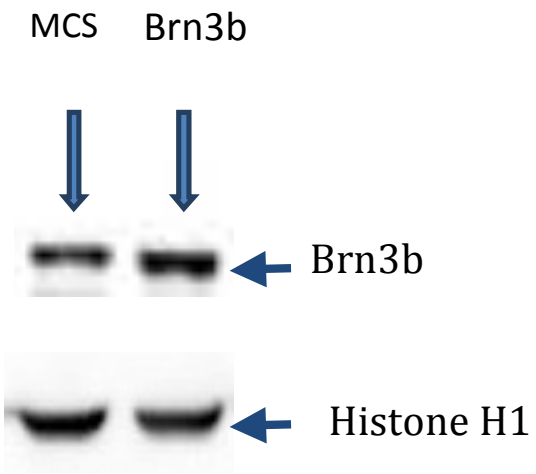


Figure 1. Immunoblot analysis of transcription factor Brn3b in 661W cells transfected with the empty vector (AAV-MCS) or the expression vector encoding transcription factor Brn3b (AAV-Brn3b). The left lanes depicts 661W cells transfected with AAV-MCS (Labeled MCS) and the right lanes represent 661 W cells transfected with the AAVBrn3b construct (labeled Brn3b). Approximately 40 µg of nuclear extracts were used for immunoblot analysis of Brn3b (top lanes) and the blots were normalized with an antibody against Histone H 1 protein to ensure equal loading of the lanes.

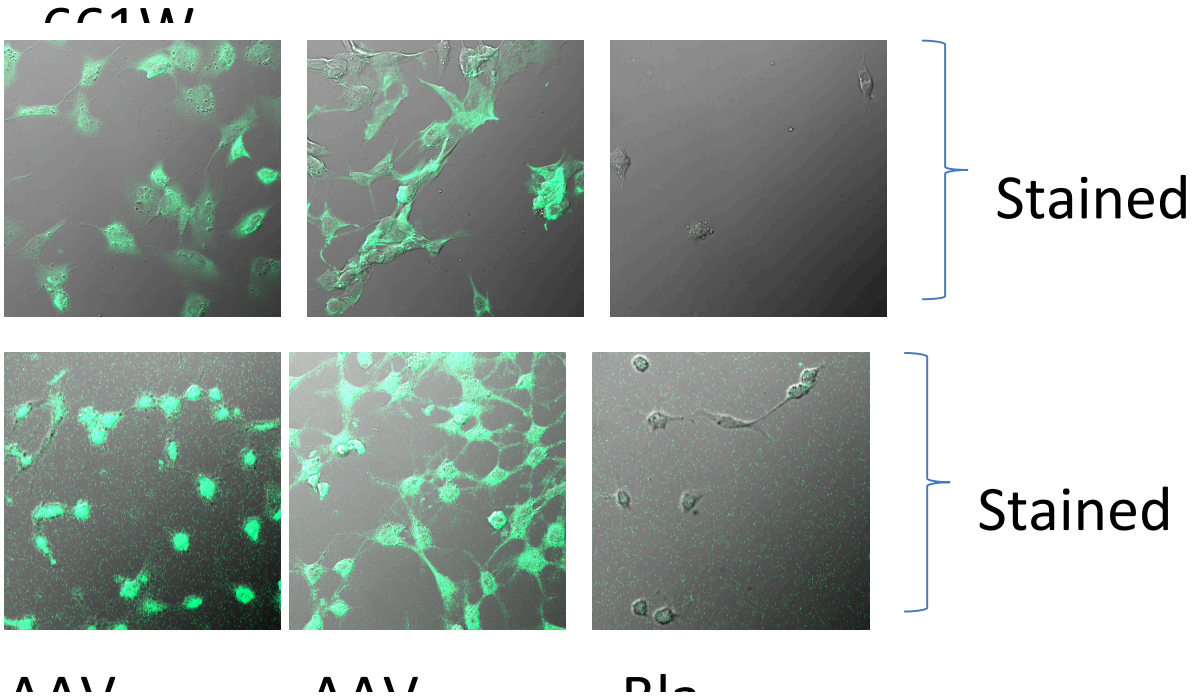


Figure 2. Immunohistochemical analysis of Brn3b and GAP43 in 661W cells transfected with AAV-Brn3b. 661W cells were transfected with either AAV-MCS (empty vector) or with the expression vector encoding transcription factor Brn3b (AAV-Brn3b). After transfection, the cells were fixed and immunocytochemistry was performed using primary antibodies against Brn3b (Top Panels) and GAP43 (Bottom Panels) followed by incubation with the appropriate Alexa488-conjugated secondary antibodies. Confocal microscopy was performed and the DIC and fluorescent images were merged to indicate the stained regions of the cells. Blank samples in which immunocytochemistry was performed with the exclusion of the primary antibody, showed minimal non-specific staining.

KEY RESEARCH ACCOMPLISHMENTS:

- Recruited and hired new Asst. Professor, Dr. Marina Gorbatyuk, who is an expert on gene therapy for retinal degeneration and is studying the unfolded protein response as a therapeutic target for the VISION project.
- Hired 6 new postdoctoral fellows to run each of the three models (3 postdocs) and to work on individual PIs on neuroprotective approaches (3 postdocs). Hired 3 new research technicians to help run the mouse models and perform histological assessment of injury-induced damage to eye and brain. In addition, 5 graduate students are currently working on this project.
- Set up a CORE laboratory responsible for establishing and running each of the 3 mouse models of injury to the visual axis and for histological assessment of damage to the retina, optic nerve, and visual axis in the brain (i.e. superior colliculus). The animal model core is supported by 3 postdocs and 2 research technicians. The histology core has a dedicated full time histology technician to process, section, and stain eye and brain sections.
- Set up independent and dedicated data management system with central server and 8 workstations to manage mouse colony, plan experiments, archive all data, and keep all members of the VISION team informed.
- Successfully established the 3 mouse models of injury (optic nerve crush, chronic ocular hypertension, and retinal ischemia/reperfusion).
- Successfully established quantitative methods to assess damage to the retina (RGC counts in retinal flatmounts, retinal layer thickness in retinal cross sections, optic nerve damage score, and neuronal cell counting is cortex of superior colliculus).
- Demonstrated statistically significant increased intraocular pressure and damage to the optic nerve in the pressure-induced mouse model of ocular injury.
- Demonstrated time dependent and statistically significant damage to retinal ganglion cells and neurons in the superior colliculus in the mouse model of optic nerve crush.
- Identified the parameters needed to develop ischemia/reperfusion damage to the retina.
- Developed the techniques to isolate high quality RNA from retinas, optic nerves, optic nerve heads, and superior colliculi of individual mice. Initiated the genomics study to identify pathogenic pathways involved in damage to these tissues in the optic nerve crush and pressure-induced damage models.
- Isolated purified mouse retinal ganglion cells and showed that sigma-1 receptor agonist agonists protect these cells from ischemic damage and promote dendritic spine outgrowth.
- The retinal ganglion cell transcription factor Brn3b was cloned into an expression vector and transfected into a retinal cell line. The enhanced Brn3b expression stimulated neurite outgrowth suggesting the potential of this factor for neuroregeneration.
- Two new non-feminizing neuroprotective estrogens had significant neuroprotective activity in an in vitro model (retinal cell line 661W) of glutamate induced cytotoxicity. This class will be tested for neuroprotective activity in our models of ocular injury.
- In vitro and in vivo proof of concept studies targeting protein stress have been validated and viral vectors are being prepared to determine the neuroprotective efficacy of these agents in our 3 models of injury.

REPORTABLE OUTCOMES:

We currently have no reportable outcomes because we spent the majority of this first year hiring scientists to support the project, setting up the animal and histology CORE, establishing the 3 models of ocular and brain injury, and developing reproducible and quantitative methods for assessing the levels of ocular and brain damage for all 3 models.

CONCLUSION:

We have made significant progress in the first year of this ambitious VISION project and have accomplished all objectives on our workplan for year 1. In this first year of the VISION project, we have established the infrastructure required to accomplish our overall goal of discovering strong neuroprotection candidates to protect the delicate neurons in the visual axis. We have recruited knowledgeable and highly trained scientists who are dedicated to this project. We have established a data management system that allows the planning and execution of all experiments, data archival and analysis, and up to the minute status of all studies for all investigators involved in this project. We have set up and validated all 3 proposed mouse models of damage to the visual axis and developed quantitative techniques to histologically assess damage to the retina, optic nerve, and visual center in the brain (superior colliculus). We have tested 4 different neuroprotective strategies in cell culture modles and are now ready to test these neuroprotectants in all three models, which will be done in the second year of this project. In addition, we have begun a large genomics study to identify the pathogenic pathways involved in damaging the visual axis of the eye and brain for each model in order to identify new therapeutic approaches. We are now situated to make significant discoveries on neuroprotection of the visual axis, which will increase our scientific understanding of the damage mechanisms involved and generate candidates to be considered for treating traumatic injury to vision in our warfighters.

The first year of this progress focused on establishing the personnel and infrastructure to accomplish our overall goal. We needed to establish all 3 injury models and develop quantitative methods to assess damage. Therefore, presentation and publication of our experimental results will not occur until the subsequent years of this project.

REFERENCES: None

APPENDICES: None